**Transcriptomic Mapping of the 5-HT Receptor Landscape**

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# Abstract

# Introduction

# Results

**Transcriptomic overview of 5-HT receptors landscape**

We analysed the single-cell scRNA-seq dataset provided by the Allen Institute {Yao, 2023 #2828} focusing on the expression of Htrs RNA across approximately 4 million brain cells. The scRNA-seq dataset comprehensively encompassed all known 14 Htr subtypes. 65.84% of cells expressed RNA of at least one Htr. Prevalence of Htrs across the entire dataset was considerably different ranging from 0.09% of Htr3b to 34.26% of Htr1f (Figure 1a). RNA of 6 Htr was found in less than 2.5% of the cells (Htr1d, Htr2b, Htr3a, Htr3b, Htr5b, Htr6). On the other hand, RNA of Htr1f, Htr2a and Htr2c was present in at least 1 every 5 cells. Average amount of RNA expression also varied across receptors (Supplementary Figure 1a). Interestingly, the variation in amount of RNA shared around half (r²=0.55) of the variability with the prevalence, i.e., genes found in more cells also tended to be expressed more at the single cell level. Beside the prevalence and amount of expression, also the distributionacross the brain was considerably different. This is exemplified by looking at the distribution of the Htr1 and Htr2 families on the uniform manifold approximations and projection (UMAP, Figure 1b). The UMAP was color-coded according to neighborhood metadata. Neighborhoods were defined both by location and neurotransmitter (Supplementary Figure 1b, Table 1). When looking at the UMAP distributions for individual Htr, considerable differences were present also within each family of receptors (Supplementary Figure 2). We analyzed these differences grouping cells by neurotransmitter, neighborhoods or class. These categorizarions divided cells in a higlhy skewed manner (Supplementary Figure 1c), for example when looking at groups by neurotransmitter release, 3 groups (Glut, Gaba and unassigned) made up for almost the totality of cells (98.47%). Expectedly, the vast majority of cells was classified as excitatory (Glut, 50.79%) and around 1 every 5 cells was found to release GABA (20.62%). All the other neurotransmitter were found in less than 1% of the cells, in particular, 5-HT releasing neurons (Sero) were found in only 0.04% of the cells. Pattern of Htrs expression across different neurotransmitter groups exhibited a relatevely high Pearson correlation coefficient (r=0.6±0.03). Sero and cholinergic neurons (Chol) showed the most distinct patterns of expression with respectively r=0.32±0.04 and r=0.37±0.05 (Figure 1c). To better evaluate the uniqueness of Htrs RNA expression per group we employed a Random Forest Classifier aiming at decoding the grouping variable from the Htrs expression. Overall accuracy of the model was 36.52%. Reflecting the correlation analysis, the confusion matrix showed that Sero and Chol were among the groups with higher true positive (TP) rate (Sero=80.82%, Chol=40.92%). Cells not expressing any neurotranmitter were also identified succesfully (81.22%). Moreover, Noradrenaline (Nora) and glycine (GABA-Glyc) releasing neurons were identified at considerable levels (Nora=48.39% and GABA-Glyc=42.74%). To understand the contribution of each Htr in each prediction we calculated the mean absolute SHAP (SHapley Additive exPlanations) values for each receptor and neurotransmitter {Lundberg, 2017 #2921; Lundberg, 2020 #2922}. The SHAP values in association with the mean prevalence enable us to understand the defining features of each group. Here we can see, for example, that the identification of Sero neurons is determined mainly by expression of Htr1a and Chol neurons by Htr4 and Htr5b. Crucially, absence of expression can also contribute to the classification, e.g., Cells not expressing any neurotransmitter are identified mainly by absence of any Htr. When looking at different neighborhoods the accuracy of the model was 51.65%. The model could differentiate best the NN-IMN-GC, TH-EPI-Glut and Pallium-Glut groups (NN-IMN-GC=73.95%, TH-EPI-Glut=66.32% and Pallium-Glut=56.99%, Supplementary Figure 3a). NN-IMN-GC includes all the cells not releasing any neurotransmitter, their classification is therefore predictably influenced by absence of any Htr. On the other hand, TH-EPI-Glut cells were characterized by the unique combination of high expression of Htr7 and low expression of Htr2a and Htr4, Pallium-Glut cells, instead, exhibited relatively low levels of Htr2c and Htr7. Notably, Htr7 and Htr1f seemed to follow opposite gradients across neighboroods.Across classes, differences in Htrs expression were more striking (Figure 1d). 6 groups could be identified with a TP rate >40%: 04 DG-IMN Glut, 05 OB-IMN GABA, 09 CNU-LGE GABA, 18 TH Glut, 22 MB-HB Sero, and 25 Pineal Glut (Supplementary Figure 3b). 04 DG-IMN Glut were charachterized by high expression of Htr4 and absence of the usually prevalent Htr2c. Similarly, 05 OB-IMN GABA cells showed virtual absence of Htr2c as well as low Htr4 and high Htr1f expression; 09 CNU-LGE GABA cells showed high Htr1b and low Htr7/Htr1a; 17 MH-LH Glut exhibited high levels of Htr5b and Htr4; 18 TH Glut showed high levels of Htr7 and virtual absence of Htr4; 22 MB-HB Sero, miroring the results showed by Sero neurons, were charachterized by high levels of Htr1a; at last, 34 Immune cells were identified by absence of any Htr expression. The use of only Htrs expression reached a unexpected 13.28% accuracy in decoding classes. Correlation between Htrs expression across the totality of cells ranged from -0.03 (Htr1f-Htr3a) to 0.311 (Htr4-Htr2c). Considerable correlation was also found for the Htr7-Htr2c (r=0.264) and Htr1f-Htr2a (r=0.212) pairs (Figure 1e). Interestingly, correlation patterns were not stable across neighboroods (Supplementary Figure 4a). For example, Pallium-Glut exhibited a negative correlation between Htr4-Htr2a not evident from the entire dataset. Of note, TH-EPI-Glut showed the highest absolute correlation across all neighboroods with r=0.609 between Htr5b-Htr4 and a unique negative correlation between Htr4-Htr7. To explore the underlying causes of the correlations we analyzed colocalization between Htrs using the same stringent threshold used by the original authors to determine neurotransmitter expression {Yao, 2023 #2828}. Across the entire dataset we observed that the most expressed genes, Htr1f and Htr2c, were often colocalized with other genes (Figure 1g). This was a driving factor for correlation. Looking more in detail across neighboroods, also here we noticed important differences, mainly explainable by differential prevalence. 86.41±1.69% of cells indeed expressed at least 2 Htrs (Figure 1f), therefore, only rarely a cell was found to express uniquely one Htr. Surprisingly, 22.88±1.9% of cells expressed at least 5 Htrs. The extensive expression across different Htr classes and the considerable coexpression within cells point at the complexity of the 5-HT system even at the single cell dimension. To facilitate an understanding of the downstream cellular effects of 5-HT we aggregated receptors according to their main intracellular effector. We aggregated Htr1 and Htr5 due to their inhibitory effect (cAMP decrerase); Htr4, Htr6 and Htr7 because of the shared downstream effect of increasing cAMP; Htr2 is the only one that causes an Ca2+ increase while Htr3 is the only ionotropic receptor. For each cell we determined the principal pathway activated by 5-HT by analyzing the amount of RNA for each Htr and, afterwards, we grouped cells across different neighborhoods (Figure 1h). Ht3 were present only in a small minority of cortical inhibitory neurons. In the telencephalon, the absolute majority of both Pallium-Glut and Subpallium-Gaba cells were linked to Htr1/5, and around one quarter of cells instead featured Htr2 as primary effector. Subcortical cells exhibited a more balanced partition without any absolute majority and a considerable presence of Htr4/6/7. In the following sections we will take a deeper look at Htrs grouped by effector and we will take advantage of the spatial information provided by the MERFISH dataset of {Zhang, 2023 #2887} regarding 9 Htrs.

**Htr1 & Htr5**

Receptors belonging to these two families have an inhibitory effect on the host cell, they are coupled to Gᵢ and cause a downstream decrease of cAMP and activation of GIRK channels {Sharp, 2020 #2888; McCorvy, 2015 #2889}. Htr1a RNA have a stable prevalence of ≈10% across neighborhoods in the RNA-seq dataset (excluding non-neuronal cells and immature neurons), with virtual absence in the TH-EPI-Glut group (Figure 2a). Htr1a co-localized most frequently with Htr1f, Htr2c and Htr2a (Figure 2b) and only in a minority of cases was expressed alone (<10%). Expression across classes was highly correlated between the RNA-seq and MERFISH datasets (Figure 2a) and show an almost perfect proportional relationship. Highest expression was found in 5-HT neurons of the mid- and hindbrain (class 22 MB-HB Sero, Figure 2c), nonetheless, cortical excitatory neurons (01 IT-ET Glut) had the higher absolute number of cells expressing Ht1a. To pinpoint the spatial location we first identified the clusters highly enriched with Htr1a RNA with a threshold of 70%, i.e., to be classified as enriched at least 70% of cells in a cluster must express the receptor. Only 6.52% of Htr1a expressing cells were contained in enriched clusters, pointing at a relatively low importance in the clustering algorithm. Looking at the spatial distribution across divisions, the highest prevalence was found in the pallidum (PAL) and hippocampus (HPF), two telencephalic regions (Figure 2d). At a more granular level, the highest prevalence was observed in the dorsal raphe (DR). DR expression is reflection of the high prevalence in Sero neurons outlined above, DR contains a substantial proportion of Sero neurons. The hippocampal structure exhibiting the higher prevalence was medial entorhinal cortex (ENTm) while the medial septum nucleus (MS) and the diagonal band nucleus (NDB), two structures linked to generation of theta waves {Winson, 1978 #2908}, contributed substantially to the expression in PAL. Levels of of expression were stable across the anterior-posterior axis like in most other Htrs (Figure 2e-f).   
Htr1b exhibited a more diverse pattern of expression across neighboroods (Figure 3a) ranging from 10 to 30%. Highest prevalence was observed in the MB-HB-Glut-Sero-Dopa group, i.e., glutamatergic, serotonergic and dopaminergic neurons located in midbrain and hindbrain. Colocalization showed a similar pattern compared to Htr1a (Figure 3b) and also here only a minority of cells expressed Htr1b alone (<10%). Looking at expression across classes, the 09 CNU-LGE GABA class showed the highest prevalence (58.06%) closely followed by 22 MB-HB Sero (53.73%) (Figure 3c). High expression in 09 CNU-LGE GABA was in sharp contrast with Htr1a that showed only minimal expression in this class (1.61%). Also in this case, and similarly to the majority of Htrs, 01 IT-ET Glut exhibited the highest absolute number of Htr1b expessing cells. 17.48% of Htr1b expressing cells belonged to highly enriched clusters and the striatum (STR) showed by far the highest prevalence with >30% (Figure 3d). Caudoputamen (CP), Nucleus accumbens (ACB), olfactory tubercle (OT), lateral septal nucleus (LSc) and the parabigeminal nucleus (PBG) all exhibited a prevalence of >30%. Distribution across the antero-posterior axes reflected the high prevalence in STR (Figure 3e-f).   
Htr1d was expressed at a much lower level, never exceeding 7% prevalence in any neighborhood (Supplementary Figure 5a). It colocalized at highest levels with Htr2c and Htr1f (Supplementary Figure 5b) and only rarely was expressed alone (<5%). Similarly to Htr1b, expression was highest in 09 CNU-LGE GABA and 22 MB-HB Sero (Supplementary Figure 5c). Notably, 09 CNU-LGE GABA exhibited the highest absolute number of cells surpassing 01 IT-ET Glut. Only a small minority of Htr1d expressing cells belonged to enriched clusters (2.08%). The paraventricular nucleus of the thalamus (PT and PVT) showed the highest prevalence at >4% (Supplementary Figure 5d-e-f).   
Htr1f showed the highest expression of all 5-HT receptors in the RNA-seq dataset. Higher prevalence was found in the Pallium and Subpallium groups (Figure 4a), reaching ≈50%. Other groups showed a prevalence of 30-40% with TH-EPI-Glut at ≈20% (Figure 4a). Htr1f was found to colocalize the most with Htr2a and Htr2c (Figure 4b). In 30% of cases Htr1f was the only Htr expressed in a cell and colocalization decreased linearly with the number of coexpressed Htrs (Figure 4b). Notably, the slope of the linear regression between values provided by RNA-seq and MERFISH was significantly lower (Figure 4c). The two datasets are, however, still highly correlated, with 66% of shared variability. Htr1f was broadly expressed across almost all classes, including some non-neuronal cells, pineal gland cells were a notable exception. In absolute numbers, cortical glutamatergic cells showed the highest expression. Spatial distribution showed a peculiarly asymettric pattern with expression concentrated in the most anterior regions. Highest expression was observed in STR, olfactory areas (OLF) and the cortical subplate (CTXsp) reaching >20% (Figure 4d). Specifically, highest expression was observed in nucleus accumbens (ACB) and olfactory tract (OT), similarly to Htr1b. The accessory olfactory bulb (AOB) was the OLF structure with the highest prevalence. Claustrum (CLA), on the other hand, was the CTXsp structure exhibiting the highest prevalence. Level of expression were not linear, with a clear peak in the frontal olfactory areas (Figure 4e-f).   
Both Htr5a and Htr5b were not included in the MERFISH dataset, therefore we do not have any direct spatial visualization of their expression. Htr5a was expressed at 8-16% prevalence across all neighborhoods (Supplementary Figure 6a) and colocalized the most with Htr1f, Htr2c and Htr2a (Supplementary Figure 6b). Expression was broadly distributed across many classes, altough only at lower levels compared to other Htrs (Supplementary Figure 6c). Only one cluster was considered enriched with Htr5a in the entire RNA-seq dataset, 3453 PAG-PPN Pax5 Sox21 Gaba. This cluster was located mainly in the midbrain reticular nucleus (RR, Supplementary Figure 6d-e).Htr5b was expressed at a much lower level (Supplementary Figure 7a), with a maximum of ≈%5 in TH-EPI-Glut. Surprisingly, even if its overall prevalence was much lower then Htr5a, 10 clusters were found to be enriched in Htr5b. This receptor was expressed at considerable levels only in the 17 MH-LH Glut class (≈50% prevalence). This was caused by high levels of expression in the medial habenula (MH, Supplementary Figure 7d-e), a structure involved in the response to stress and fear {Chou, 2016 #2913;Soria-Gomez, 2015 #2910;Winson, 1978 #2908;Yamaguchi, 2013 #2909}. Some expression was also evident in the posterior part of the brain, specifically in the inferior olivary complex (IO), a structure strongly linked to cerbellar Purkinje cells {Loyola, 2023 #2914}. This expression was driven by a single supertype, 253 IO Fgl2 Glut.

**Htr2**

The Htr2 family is mainly linked to Gq/11 and causes excitation by increasing intracellular Ca2+. Htr2a, famous for being instrumental in mediating the effects of psychedelics {Nichols, 2016 #854}, is found across the brain with highest prevalence in telencephalic neighborhoods, Pallium-Glut and Subpallium-GABA (Figure 5a). Colocalization was highest with Htr1f and Htr2c (Figure 5b). Considerable expression (≈40%) was found in 01 IT-ET Glut, 07 CTX-MGE GABA and 16 HY-MM Glut classes (Figure 5c). Htr2a was also prevalent across many other classes across the whole brain. Similarly to Htr1f, also here the MERFISH dataset hinted at a lower overall expression when compared to RNA-seq. Shared variability between the two was, nonetheless very high. CTXsp showed the highest prevalence, reaching >12% (Figure 5d). Isocortex and STR exhibited both ≈5% prevalence. At a structure level, surprisingly, two structures belonging to the mammillary complex (dorsal premammillary nucleus, PMd and tuberomammillary nucleus,TMd) were in the top ten. The mammillary complex has been linked to Alzheimer´s disease {Huang, 2023 #2915}, and memory {Roy, 2017 #2916}. CLA and the endopiriform nucleus (EPd) showed the highest absolute prevalence. Interestingly, CLA has been proposed to play an important role in mediating the effects of psychedelic compounds {Doss, 2022 #2917}. Expression of Htr2a was highest in frontal regions of the brain, decaying linearly to virtula absence in the cerebellum (Figure 5e-f).   
Htr2b was found only in a minority of neurons and was not included in the MERFISH dataset. No cluster was found to be enriched with Htr2b. Interestigly, neurons belonging to the Pineal Glut class showed the highest prevalence at 7.34% (Supplementary Figure 8c).   
Htr2c was found at highest prevalence in the MB-HB-Glut-Sero-Dopa and Hy-EA-Glut-Gaba groups (Figure 6a). With the exception of Pallium-Glut, its prevalence was always >40%. Colocalization was highest with Htr1f, Htr4 and Htr7 (Figure 6b). In similar fashion to Htr2a and Htr1f, also here there were discrepancies between the RNA-seq and MERFISH methods(Figure 6c). Expression was broadly distributed across many different classes, also subcortically, with the exception of pineal gland cells. Many classes exhibited a prevalence >60%. The majority of cells expressing Htr2c belonged to enriched clusters. Highest prevalence was found in STR. Similarly to Htr1b, ACB, CP and OT exhibited the highest prevalence (Figure 6d-e-f).

**Htr4, Htr6 and Htr7**

These receptor are all connected to Gs {McCorvy, 2015 #2889}, leading to increasing cellular levels of cAMP and excitation. Htr4, similarly to Htr2c, showed highest prevalence (>40%) in the MB-HB-Glut-Sero-Dopa and Hy-EA-Glut-Gaba groups (Figure 7a). It colocalized the most with Htr2c and Htr1f (Figure 8b). Discrepancies in amount of expression between RNA-seq and MERFISH were present also here (Figure 7c). This did not affect significantly, however, the correlation between the two datasets. Expression across classes was broadly distributed, with many classes showing a prevalence >40%. In absolute numbers, expression in excitatory cortical neurons was comparable to other classes. Spatial distribution exhibited a peculiar pattern with high prevalence in one specific structure of the STR: OT (Figure 7d-e-f). A subclass of interneurons present in OT (060 OT d3 Folh1 Gaba) showed a >98% prevalence. PAL and HPF also exhibited relatively high prevalence (≈10%). Dentate gyrus (DG) granule cells (037 DG Glut) were the main driver of the high prevalence.   
We do not have MERFISH information about the rarely expressed Htr6 and no enriched cluster was present in the RNA-seq dataset. The 09 NU-LGE GABA class exhibited the highest prevalence with 7.73, still, the absolute majority of neurons expressing this gene were excitatory cortical neurons (Supplementry Figure 11c).   
Conversely, Htr7 was expressed in >10% of the totality of cells. It reached ≈60% in the TH-EPI Glut group, and considerable amounts (≈40%) in MB, HB and HY groups (Figure 8a). Colocalization was the highest with Htr2c and Htr1f (Figure 8b). Expression was broadly distributed across classes present in HY, MB and TH (Figure 8c). It colocalized the most with Htr2c, tr1f and Htr4. Htr7 was broadly expressed across classes, especially in subcortical structures. Peak prevalence was found in 10 LSX GABA and 16 y MM Glut with >60%. Htr7 enriched clusters were located mainly in HY and TH (Figure 8d). At a structure level, the parafascicular (PF) and paraventricular nucleus (PVT) of TH showed the highest prevalence (>30%).

**Htr3**

The Htr3 family is the only ionotropic Htr and it causes direct excitation by allowing the influx of cations. The Htr3a subunit is required for the formation of a functional channel {Maricq, 1991 #2918} and can form functional homopentameric receptors {Walstab, 2010 #2919}. Heteromeric receptors containing Htr3b have an increased channel conductance and different selectivity {Davies, 1999 #2920}. Htr3a is expressed almost uniquely in the Subpallium-Gaba neighborood, with a prevalence of ≈8% (Supplementary Figure 10a), specifically in the 06 CTX-CGE GABA class (Supplementary Figure 10c). It is one of the few Htr, together with Htr3b and Htr1d, that is not expressed the most in absolute numbers in 01 IT-ET glut. It colocalizes mainly with Htr2c and Htr7 (Supplementary Figure 10b). This Htr was mainly expressed in OLF, CTXsp, HPF and Isocortex (Supplementary Figure 10d) and is most prevalent in the anterior part of the brain, altough, puzzingly, with slightly lower level of expression (Supplementary Figure 10e-f). Htr3b was not included in the MERFISH dataset and no cluster was found to be enriched with this receptor. Htr3b was the least expressed Htr gene in the entire dataset. Similarly to Htr3a, its expression was delimited to the 06 CTX-CGE GABA class (Supplementary Figure 11c).

# Discussion

# Materials and Methods

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**Htr1a**

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# Data and materials availability

All the code used to process the dataset is available at https://github.com/RobertoDF/Transcriptomics-5-HT, pre-computed data structures can be downloaded at 10.6084/m9.figshare.20209913. All figures and text can be reproduced using code present in this repository. Access to the original datasets is provided by the Allen Institute at https://portal.brain-map.org/atlases-and-data/bkp/abc-atlas.

# Contributions

Conceptualization, data curation, formal analysis, investigation, visualization: RDF. Writing - original draft: RDF. Writing - review & editing: RDF, DS. Funding acquisition: DS.

# Figures

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**Htr1a**

# Supplementary Figures

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